Committee on the Use of Biotechnology Research in Alfalfa Improvement
Charlie Brummer, Yves Castonguay, Deborah Samac (Chair), Stephen Temple

Preamble

The 2004 report was compiled from responses received from inquiries regarding biotechnology research at laboratories around the world. The report is organized geographically. The names and addresses for a contact person at each location are listed at the end of the report. These individuals are identified by a* in the narrative. Although we attempted to contact all labs conducting alfalfa biotechnology research, we regret any omissions that may have occurred. Please inform the committee of omissions so that the next report will be complete.

United States (compiled by Charlie Brummer and Stephen Temple)

Arizona State University

Ilga Winicov* and her group have focused on characterization of genes over-expressed in salt-tolerant alfalfa (*Medicago sativa)*. Salt-tolerant cell lines were originally selected in tissue culture and regenerated to provide the salt-tolerant plants. Two genes of particular interest have emerged from this work: 1) *Alfin1,* a novel zinc finger transcription factor and 2) the *MsPRP2* gene which encodes a proline-rich cell wall protein. Both genes are expressed in a root-specific manner and have been used to establish that *Alfin1* expression is essential for root growth and *Alfin 1* over-expression results in both increased root growth and increased salinity tolerance. Alfin1 is a DNA binding protein. It binds to the *MsPRP2* promoter and up-regulates expression from this root-specific promoter. The properties of the transcription factor and the promoter have been exploited for expression of heterologous genes in alfalfa.

Forage Genetics International

The biotechnology research program at Forage Genetics International (FGI) is under the direction of Stephen Temple* and Mark McCaslin*. The primary focus of the research program continues to be the development of Roundup Ready alfalfa. FGI in collaboration with Monsanto is seeking regulatory approval for the commercial release of Roundup Ready alfalfa in the USA and key export markets. This is anticipated to occur in 2005. Agronomic evaluation of the first generation Roundup Ready experimental varieties continues at over a dozen locations in the USA. Roundup Ready alfalfa provides an excellent weed control option. Molecular markers developed by the FGI biotechnology program and Monsanto have been instrumental in the development of the Roundup Ready experimental varieties that achieve the required high levels of trait purity. These markers and the systems developed to utilize them have overcome the unique challenges of working with an out-crossing tetraploid crop species. Additional alfalfa biotechnology research being carried out by FGI scientists is in collaboration with Richard Dixon* and colleagues at the Samuel Roberts Noble Foundation and Ron Hatfield* and colleagues from the US Dairy Forage Research Center under the newly formed Consortium for Alfalfa Improvement (CAI). In 2003 FGI established a large field evaluation of transgenic plants containing constructs designed to reduce lignin levels. The preliminary results of this study are presented elsewhere in the NAAIC proceedings. Other CAI umbrella projects where FGI has active research interests include the development of bloat-safe alfalfa. This is being accomplished by engineering the tannin biosynthetic pathways for constitutive or foliar tannin ex-pression. FGI is also collaborating with Margaret Gruber*, Agri-Food Canada on specific aspects of the alfalfa tannin project.

Iowa State University

E. Charles Brummer* and his laboratory (Diane Luth, lab manager) are conducting genetic mapping to identify loci (QTL) controlling biomass yield and winter hardiness, as well as their physiological components in both diploid and tetraploid populations. Analyses of gene expression and the identification and characterization of genes related to winter survival are underway. In collaboration with Jeff Doyle at Cornell University, they are investigating the systematics of the *M. sativa/falcata complex* and in particular the evolution of polyploidy and domestication.

New Mexico State University

Ian Ray* and Tracy Sterling are collaborating with Mary Sledge* (Samuel Roberts Noble Foundation) to identify physiological, biochemical, and genetic mechanisms used by alfalfa progeny that are most successful under drought and salt-stress. Microarray technologies available in *Arabidopsis* and *M. truncatula* have been utilized to identify over 500 drought-responsive and 600 salt-stress responsive genes in alfalfa leaves and roots. Northern analyses have confirmed most microarray results. Candidate genes identified were involved in carbon and nitrogen assimilation, lipid metabolism, antioxidant defense, photorespiration, electron transport, amino acid biosynthesis, osmotic adjustment, intra- and inter-cellular transport, signal transduction and gene regulation. Nucleic acid sequencing of candidate alfalfa cDNAs detected >71% and >85% identity with their stress-responsive elements on the *Arabidopsis* and *M. truncatula* arrays, respectively. BLAST analyses of the *M. sativa* sequences agreed with the microarray annotations. Over 100 of these genes are being converted into polymorphic markers suitable for mapping/QTL association analyses using a procedure that incorporates gene-specific primers and amplified fragment length polymorphisms. These markers will be used for selective genotyping analysis in tetraploid alfalfa linkage mapping populations that are segregating for either drought or salt tolerance. Two mapping populations will be evaluated under limited irrigation during 2005-2007 in New Mexico and Oklahoma. Two additional populations will be evaluated for segregation of the salt tolerance phenotype in a greenhouse environment. The significance of marker-trait associations will be determined. This type of genetical genomics approach will provide crucial verification of which candidates actually influence/control the tolerant phenotype. Physiological traits will also be measured on those families possessing extreme phenotypes for the traits of interest.

Champa Sengupta-Gopalan*, Suman Bagga, Jose Luis Ortega, Carol Potenza and other members of the lab are working in the general area of metabolic engineering with regards to nitrogen and sulfur metabolism. The research program uses molecular, biochemical, physiological and cellular approaches. A concerted effort is being made to understand the regulatory mechanism underlying the expression of the enzyme glutamine synthetase (GS) and using this understanding to manipulate GS levels in a tissue-specific manner in transgenic alfalfa. The ultimate goal of this project is to improve nitrogen use efficiency in alfalfa and other forage legumes. Towards achieving this goal, work is also being done to understand the role of the regulatory protein, PII (C/N sensor) and the effect of over-expressing this gene in alfalfa. The project is also looking at the effect of over-expressing sucrose phosphate synthase on the expression of GS. More recently, projects have been undertaken to look at the effect of over-expressing chloroplastic glutamine synthetase on drought and salinity stress tolerance in alfalfa. Another major project in the lab is to increase the methionine content in the vegetative tissues of alfalfa to produce animal feed that is balanced in its amino-acid content. The experimental approach has been to simultaneously express genes encoding for high methionine protein (corn seed storage proteins-zeins) and a gene for a key enzyme in methionine biosynthesis, cystathionine gamma synthase. Work is in progress to manipulate other key enzymes in the methionine metabolic pathway and this includes down-regulating threonine synthase and S-adenosyl methionine synthase. More recently, the lab is focusing on subjecting the different transgenic plants produced for the different projects to microarray analysis, proteomics and metabolite analysis.

Purdue University

Jeff Volenec* and his laboratory (Suzanne Cunningham, W. Kess Berg, Khaldoun Al-Hadid, Jamalyn Evans) continue their research to identify and characterize physiological, biochemical, and molecular mechanisms influencing alfalfa growth and stress tolerance. Four research topics are currently being examined in detail:

(1) Characterize mechanisms controlling synthesis and degradation of organic reserves (starches, sugars, and storage proteins) in legume roots, and understand the role of organic reserves in shoot growth and stress tolerance. Genes for three of the four vegetative storage proteins found in alfalfa taproots have been cloned and their characterization is underway.

(2) Understand physiological and molecular factors controlling crown bud dormancy and development, and their impact on shoot development and forage yield. Defoliation-induced changes in bud gene expression are being evaluated. Dormant and non-dormant germplasms sampled both in summer and autumn are being evaluated.

(3) Determine physiological and molecular basis of fall dormancy and its association with alfalfa winter hardiness. Expression of genes associated with fall dormancy and winter survival is unaffected by fall cutting management that reduces winter survival. Accumulation of sugar in taproots of plants defoliated in autumn is greater than that of plant left uncut even though the latter plants have superior winter survival. Understanding the physiological and molecular factors regulating winter survival remains a priority.

(4) Characterize how potassium (K) and phosphate (P) nutrition alter physiological and biochemical processes in roots that ultimately improve alfalfa stress tolerance and growth. Contrary to our expectations, alfalfa death was independent of K nutrition, and increased by P fertilization. Stand losses occurred over summer and not winter. Fertilization with P without K accelerated stand decline. Studies are underway to learn how nutrition impacts root physiology and expression of key genes associated with alfalfa persistence.

The Samuel Roberts Noble Foundation:Plant Biology Division, Ardmore OK

Richard Dixon*, his colleagues and numerous collaborators are currently engaged in numerous research projects targeted towards alfalfa improvement. A key project continues to be deciphering the complex networks in monolignol formation, phenylpropanoid coupling and lignin assembly with the goal of developing forage crops with improved digestibility. The main objective is to launch a comprehensive study on lignin assembly, and the effects of its genetic manipulation. A major emphasis of the research is to systematically down-regulate each and every step in both monolignol formation and subsequent coupling in alfalfa. Analysis of these transgenic plants will combine proteomics and genomics with a very detailed analysis of phenylpropanoid pathway metabolites (e.g., by metabolic profiling), as well as the most comprehensive analyses of lignin macromolecular assembly undertaken thus far. This will enable identification and dissection of the various individual metabolic networks involved in forming the three monolignols (and related metabolites), and the precise effects of down-regulation on lignin macromolecular assembly. In addition to detailed chemical and biochemical analyses, plant tissues will also be evaluated to ascertain the effects on their mechanical properties. Through collaboration with John Ralph, U. S. Dairy Forage Research Center, Madison, WI, various NMR techniques are being applied to provide a detailed picture of the lignin structure in the various transgenic lines generated. With Lloyd Sumner's group at Noble the effects of genetic modification on the cell wall proteome are being studied. Through collaboration with Joe Noel of the Salk Institute Structural Biology Laboratory, the potential for structure-based modification of enzyme properties for

the genetic modification of lignin is being investigated. In collaboration with Hugh Aljoe, the effects of various different types of lignin modification on the in rumen digestibility of alfalfa forage will be determined. Other research projects in the Dixon laboratory include: 1. Discovery of genes involved in the control of secondary product synthesis in *Medicago*. This research has applications for production of antimicrobials and insecticidal compounds in plants. 2. Genetic modification of health-promoting isoflavones in alfalfa. This work will allow the testing of the hypothesis that isoflavones have health beneficial effects for humans. 3. Functional genomics of triterpene saponin biosynthesis in *Medicago truncatula*. The applications of this work are to improve the palatability and pest resistance in alfalfa. 4. Proanthocyanidin biosynthesis in *Medicago* species. The goals of this project are the development of bloat-safe alfalfa and the development of new foods with enhanced antioxidant potential for disease prevention.

The Samuel Roberts Noble Foundation: Forage Improvements Division, Ardmore OK.

Mary Sledge*, Zengyu Wang, and Joe Bouton* of the Noble Foundation's Forage Improvement Division are working to develop improved cultivars of alfalfa for use in the southern Great Plains region. The work is also collaborative with Richard Dixon* and Greg May of the Foundation's Plant Biology Division. The main target traits are aluminum (Al) tolerance, drought tolerance, and cotton root rot resistance. In the case of Al tolerance, DNA markers have been identified and are being used to select for plants with improved Al tolerance, which will then be validated through field trials. To complement this work, genomics techniques are also being used to isolate and identify genes from the model species, *M. truncatula*, for Al tolerance. This should result in better tools to improve alfalfa for Al tolerance, and could validate the current research focus on model organisms, paving the way for discovering other genes of agronomic importance in *M. truncatula* that could be used for alfalfa improvement. In collaboration with Ian Ray, New Mexico State University, markers are being identified for drought tolerance and a genetic map is being constructed. Drought tolerance field trials will be planted in fall 2004. In the following years, we will be able to associate specific DNA markers with multiple components of drought tolerance. Finally, a new project just getting underway is using a transgenic approach to insert gene(s) for production of condensed tannins in order to reduce pasture bloat and improve nutritive quality.

Southeastern Oklahoma State University

Nancy L. Paiva* is continuing the characterization of *Medicago truncatula* beta-glucosidases highly specific for the hydrolysis of isoflavonoid glucosides, and is determining the potential of these beta-glucosidases to improve human nutrition. These isoflavonoid derivatives and enzymes are also found in alfalfa (*Medicago sativa)*, where they are thought to be important in plant defense and establishment of beneficial symbioses. Additional funding has been received recently to pursue the isolation of clones encoding new isoflavonoid biosynthetic enzymes and to use microarray technology to study the expression of genes related to the biosynthesis of alfalfa metabolites potentially beneficial to human health.

USDA-ARS, Beltsville Agricultural Research Center, Beltsville, Maryland

T. Austin Campbell* has mined *Medicago truncatula* EST and BAC library databases for Simple Sequence Repeats (SSRs) DNA markers appropriate for mapping in autotetraploid alfalfa. Approximately 1000 EST and 500 BAC SSRs have been identified. So far, 116 EST-SSR markers have been added to a molecular map of an alfalfa population segregating for biomass yield, autumn growth and winter survival. Eight consensus linkage groups were identified and the map span is approximately 673 cM with 3.9 cM between loci. Gary Bauchan* and post doctoral associate Chunlin He have developed a tetraploid alfalfa genomic library and identified 118 genomic SSRs from this library. Of the 118 SSRs, 61 proved to be polymorphic among the nine historically recognized alfalfa germplasm

sources, two very non dormant alfalfa germplasms, diploid *M. sativa* ssp. *coerulea* and ssp. *falcata* and *M. truncatula*. Using cluster analysis and multivariate analysis we were able to distinguish: diploid *ssp. falcata*; *M. truncatula* ('Jemalong'), Ladak ('Ladak'), very non dormant (UC-1465), Indian (Sirsa Type 9), Flemish ('Dupuit'), Peruvian (Hairy Peruvian), African 2 ('Moapa'') and Turkistan (Kayseri). Additional genomic SSRs are being developed and EST derived SSRs are being utilized to distinguish these alfalfa cultivars.

U. S. Dairy Forage Research Center, Madison, WI

The laboratories of Michael Sullivan* and Ronald Hatfield* are using transgenic alfalfa as a model system to characterize polyphenol oxidase-mediated inhibition of post-harvest proteolysis. Because alfalfa leaves seem to have little if any polyphenol oxidase (PPO) activity or *o*-diphenol PPO substrates, alfalfa is an excellent model system for analyzing the various components involved in this enzyme/substrate system. In collaboration with Deborah Samac* (USDA-ARS, St. Paul, MN) and Richard Muck (U.S. Dairy Forage Research Center), transgenic alfalfa expressing a red clover PPO cDNA was used to demonstrate that the abundant PPO and *o*-diphenols present in red clover leaves are responsible for decreased post-harvest protein breakdown seen for that forage crop upon ensiling. The group is currently using the transgenic alfalfa system to characterize PPO proteins with respect to substrate specificity, latency, and protein structure/function relationships; elucidate the biochemical mechanism of PPO-mediated proteolytic inhibition; and optimize the system as a practical silage treatment. To compliment the PPO work, the group is also taking both conventional and biotechnology approaches to increase *o*-diphenol production in alfalfa leaves, including a screen of alfalfa germplasm for natural variation in foliar PPO expression and *o*-diphenol production and identification of red clover enzymes involved in *o*-diphenol biosynthesis.

USDA-ARS-Plant Science Research Unit, University of Minnesota, St. Paul, MN

The laboratory of Deborah Samac* is engaged in several projects to develop new crop uses and improve alfalfa quality. In collaboration with Mike Sadowsky, University of Minnesota, plants are being developed for remediation of atrazine-contaminated soil and water, through expression of a bacterial gene for atrazine chlorohydrolase. Constitutive expression of a synthetic gene with plant-preferred codons results in a high level of tolerance. With Hans Jung, John Gronwald, and David Somers (University of Minnesota), tissue-specific promoters are being used to alter the content and composition of pectin in cell walls of alfalfa stems to improve forage quality. Using ESTs from *M. truncatula*, gene expression in seed coats of normal alfalfa and variants is being quantified to identify regulatory genes involved in condensed tannin synthesis. Microarrays are being used to identify genes expressed in *M. truncatula* in response to pathogens and aluminum stress.

USDA-ARS-Vegetable and Forage Crop Research Unit, Prosser, WA

George Vandemark* is continuing his research to develop and evaluate PCR-based marker systems for resistance to a variety of alfalfa pathogens. 1. Development of a multiplex real-time PCR assay for the simultaneous evaluation of bulked plant samples and individual plants for resistance to both *Aphanomyces euteiches* and *Phytophthora medicaginis*. 2. Development of a real-time PCR assay for the evaluation of bulked plant samples and individual plants for resistance to *Fusarium oxysporum*. 3. Development of a real-time PCR assay for the evaluation of bulked plant samples and individual plants for resistance to *Verticillium albo-atrum* (in collaboration with Richard Larsen, USDA-ARS, Prosser, WA). 4. Investigating genetic diversity both within and between alfalfa cultivars and germplasms with sequence related amplified polymorphisms (SRAPs) and amplified fragment length polymorphisms (AFLPs). 5. Determining gene copy number with real-time PCR.

University of California, Los Angeles

In addition to using alfalfa as a host for *Sinorhizobium meliloti* for studies of nodulation and nitrogen fixation, transgenic alfalfa (*Medicago sativa* cv. Regen) is being used for investigating the expression of various plant genes with the goal of understanding their function. W.M. Karlowski and Ann M. Hirsch* cloned an alfalfa gene encoding a small protein with a RING-H2 motif and an N-terminal trans-membrane domain. When an antisense version of this gene was introduced into alfalfa, no significant difference was seen from the control plants, whereas over-expression resulted in dramatic phenotypic changes in both transgenic alfalfa and Arabidopsis plants. A connection between the RING protein and auxin signaling is postulated. L.M. Brill, N.A. Fujishige, C.A. Hackworth and A.M. Hirsch studied the role of two of the three alfalfa lectin genes in symbiosis and found that antisensing the *MsLEC1* gene resulted in significant changes in the symbiotic phenotype of alfalfa. Earlier work demonstrated the importance of the *MsLEC1* and *MsLEC2* genes in alfalfa development and reproduction. The Hirsch (UCLA) and Hawes (University of Arizona) labs have utilized transformed alfalfa with both sense and antisense constructs of UDP-glucuronyltransferase (UGT) to achieve a better understanding of how this enzyme functions. Normally UGTs and ß-glucuronidase (GUS) function together to modulate the activity of steroid hormones in animal cells. Previous studies using the 35S CaMV promoter demonstrated that antisensing UGT in alfalfa resulted in severely affected growth and also altered response to symbiotic rhizobia and pathogenic fungi. Interestingly, experiments with localizing UGT expression utilizing GUS as a reporter resulted in lethality in pea, alfalfa and Arabidopsis. This is a cell-specific (border cell) lethal phenotype, which might be useful for investigating cell cycle control in plants. Alfalfa is being used as a model plant in ongoing studies with the Center for Dietary Supplements Research: Botanicals (UCLA) on developing forensic PCR methods with the ultimate purpose of identifying plant contaminants in dietary supplements.

Washington State University and USDA-ARS, Pullman, WA

Ted Kisha* is comparing molecular marker types for the characterization of alfalfa populations. Ninety-six plants of three alfalfa cultivars (Aragon, Turkey; Hunter River, Australia; and Yonca, Spain) were examined using SCARs of a hypervariable region of the chloroplast, RAPDs, simple sequence repeat (SSR), and AFLPs. All marker types indicated the same relative relationships among the three accessions, with Hunter River and Aragon being the most similar and Yonca being more distant from the two. A single chloroplast SCAR had a polymorphism information content (PIC) of 0.89. Simple sequence repeat (SSR) markers, RAPD markers and AFLP markers had average PIC values of 0.75 (data not complete), 0.27, and 0.36 respectively. Chloroplast SCARs and SSR markers examined one locus per gel, while RAPD and AFLP markers revealed polymorphic markers at an average of 4.3 and 28 loci, respectively. Cost per sample per locus analyzed is estimated at $1.02, $0.18, and $0.06 for SSR, RAPD, and AFLP markers, respectively.

Canada (compiled by Yves Castonguay)

Agriculture and Agri-Food Canada, Saskatoon

At the Saskatoon Research Centre, Agriculture and Agri-Food Canada, Margaret Gruber* is continuing work on developing alfalfa with reduced bloat potential, reduced greenhouse gas production, and increased protein bypass. Her group is attempting to alter the expression of genes associated with condensed tannins in several crops. Earlier, this laboratory isolated the world's first set of *myc*-like regulatory genes that affect the accumulation of condensed tannins in trefoil forage and in barley seed coat. More recently, the group has focused on using functional genomics tools in *Arabidopsis* and the trefoils to isolate additional novel biochemical genes with potential to modify tannin biosynthesis. One regulatory gene is being tested in alfalfa in combination with a newly-isolated tannin biochemical gene (leucocyanidin reductase) in a collaboration with Forage Genetics International. The maize anthocyanin regulatory gene *Lc* has also been over-expressed in alfalfa, causing the reduction of flavones in forage

and the accumulation of small amounts of condensed tannins in forage and developing seed. Studies in Tim McAllister's laboratory at the Lethbridge Research Centre indicate that the *Lc*-enhanced genotypes represent proof-of-concept that tannins in alfalfa can improve forage quality, although optimum tannin concentration has not yet been achieved. Field-grown *Lc*-enhanced forage has a lower initial rate of digestion and gas production *in vitro* compared with parental plants. Preliminary laboratory bioassays in Julie Soroka's entomology laboratory at SRC indicate that *Lc*-enhanced alfalfa reduces pea aphid viability; hence, these plants are now being tested for resistance to insects under field conditions.

Medicago Inc., Ste-Foy, Quebec

Medicago Inc. is a privately-held biopharmaceutical company pioneering the development of new-generation of biopharmaceutical products designed to fight major human diseases. Louis P. Vézina* (Vice-President, Research & Development) and his team are involved in several R&D projects. Over the years, Medicago has developed a new proprietary platform for the production of this new generation of products, combining natural characteristics of alfalfa with advanced genetic engineering techniques. More specifically, Medicago has succeeded in coupling the high flexibility and capacity of conventional plant-based technologies with the benefits of safety and control offered by producing in confined hi-tech greenhouses. Recently, Medicago evolved from a Contract Manufacturing Organization into a Product Development Organization. Two major families of biopharmaceuticals are currently being produced in alfalfa, monoclonal antibodies and plasmatic proteins. As a result, product specific approaches have been initiated to identify, characterize and control important biological processes such as recombinant protein targeting, accumulation, and proteolysis. Post-translational modification of alfalfa made pharmaceuticals is another important area of R&D with an emphasis on N-glycosylation. Research activities on core enabling technologies are still very important. Specific efforts are currently being spent on the development of high throughput methods for plant transformation, transgenic line selection and protein expression screening. The main objectives of Medicago's R&D activities are to optimize biopharmaceutical production in alfalfa and to insure product safety, homogeneity, and bio-activity. Over the last year, Medicago has committed to produce its first plant made pharmaceutical in confined hi-tech greenhouses. To meet this target, a 1300 m^2 greenhouse complex is currently being built in the Quebec City area. This complex will meet Bio-safety Level 2 (BL2) standards and house various activities including plant acclimation, conservation and scale up production. A specialized biomass treatment unit will also be part of the greenhouse complex to allow primary extraction and recovery of biopharmaceuticals. In addition to enable scale up production for specific biopharma-ceuticals, this facility will allow further optimization of alfalfa growth in confined environment in relation to light requirements, bioburden, and homogeneity of raw biomass.

University of Guelph

At the University of Guelph, Steve Bowley* and his research group are continuing their work on the modification of winterhardiness, carbohydrate profile, and studies of the heat shock response using genetic engineering technology combined with field breeding methodologies. The emphasis is on modification of environmental stress tolerance systems and carbohydrate metabolism. The work includes: 1) Production of transgenic plants and field evaluation; 2) Pyramiding transgenes using conventional breeding; and 3) Contractual transformation and evaluation of proprietary genes. Judy Strommer, Pat Shewen and Reggie Lo are testing concepts for the oral delivery of vaccines to ruminants using transgenic forages as the delivery vehicle. They are constructing chimaeric genes that encode antigenic determinants from proteins of *Mannheimia haemolytica*, a serious respiratory pathogen, and introducing them into alfalfa and clover so as to maximize their immunogenic potential. Four antigens have been produced so far, all with reasonable levels of expression in RSY27. Those tested to date have been demonstrated to be immunogenic in rabbits to be stable in dried feed stored at room temperature.

Agriculture and Agri-Food Canada, Ste-Foy, Québec

At Agriculture and Agri-Food Canada, Sainte-Foy, Québec, Yves Castonguay*, Serge Laberge, Annick Bertrand and Réal Michaud have a number of projects on biotechnology and genomic research. Activities include: 1) High throughput analysis of Expressed Sequence Tags (ESTs) isolated from cDNA libraries of cold acclimated alfalfa and functional analysis of their expression on high density grids. More than 10,000 ESTs have been sequenced and their expression is currently being studied using macroarray analysis. Identification of genes with potential applications in the improvement of persistence and forage quality is a priority. Genes closely associated with cold tolerance have been identified and will be validated in transgenic plants. 2) Characterization of genes and proteins involved in proteolysis (protease and protease inhibitors) and cell wall biosynthesis (lignin biosynthesis genes, cell wall protein and cellulase) in alfalfa in order to identify candidate genes involved in the nutritive value of alfalfa. 3) Development of alfalfa populations with improved freezing tolerance and their use for the analysis of the molecular and genetic bases of superior winterhardiness. 4) Search for candidate genes associated with cold tolerance using a bulk segregant analysis of pooled DNA samples from genotypes of alfalfa populations selectively improved for freezing tolerance. 5) Development of an integrated perspective on the effects of environmental parameters and management practices such as soil humidity, ice encasement, increased CO_2 or fall cutting schedule on molecular and genetic changes associated with the acquisition of freezing tolerance in alfalfa.

Mexico (compiled by Stephen Temple)

Nitrogen Fixation Research Center – National University of Mexico

Research in Georgina Hernández's* group is directed to studies of carbon and nitrogen metabolism in alfalfa nodules during symbiosis with *Sinorhizobium meliloti*, a nitrogen fixing bacterium. The group has used reverse genetic approaches, such as over-expression and antisense inhibition, to modulate nodule-specific gene expression of the ammonium assimilation enzymes: glutamine synthetase (GS) and glutamate synthase (NADH-GOGAT). For these projects, both alfalfa and the model system *Medicago truncatula* are being used and some of them are in collaboration with Carroll Vance's* group from the University of Minnesota-USDA/ARS. Recent projects have focused in the molecular and physiological characterization of transgenic alfalfa plants with nodule specific NADH-GOGAT inhibition that resulted in drastic deleterious effects in symbiotic N/C assimilation. A long-term goal of this research is to generate transgenic alfalfa or *M. truncatula* plants improved in symbiotic N/C assimilation.

Europe (compiled by Deborah Samac)

AgroBioInstitute, Sofia, Bulgaria

The group of Dimitar Djilianov* is working in the area of ecophysiology and abiotic stress tolerance with *M. sativa* genotypes. Several lines tolerant to osmotic stress have been selected *in vitro* after indirect somatic embryogenesis regeneration for PEG tolerance. The lines show higher abiotic stress tolerance than the explant source genotype. Their leaves showed considerable tolerance to severe osmotic stress (40% PEG for up to 72 hours). Endogenous ABA and proline concentrations, as well as the ion leakage were studied under stress conditions and after recovery. Seeds and seedlings, obtained from self-pollination showed higher salt tolerance then those of the explant source genotype. The same is true for the PSII system under drought and salt. At present, a field trial is established at the Forage Crops Institute.

The Functional Genetics group of Mariana Vlahova* is studying somatic embryogenesis and using gene transfer techniques for alfalfa improvement. Recent studies focused on molecular and cellular biology of direct somatic embryogenesis in diploid (*Medicago truncatula*, *M. littoralis, M. murex, M. polymorpha, M. coerulea*) and tetraploid *Medicago* species. The role of some growth regulators such as 2,4-D and TDZ as well as the influence of chemical and physical pre-treatment, genome size,

polysomaticy of the plant tissue, physiological state, age and endogenous phytohormone balance of the primary explant, were studied. Transgenic diploid and tetrapolid *Medicago* plants expressing the GUS gene under different cell cycle regulating genes (cyc1, cyc3, cdc2a) and the *gfp* reporter gene under the 35S promoter were produced. To produce Basta-tolerant plants, transformations of the highly embryogenic line R4 of the Bulgarian cultivar "Obnova" were performed with constructs carrying the *bar* gene. Transgene expression was demonstrated by spraying T_o and T_1 plants with the herbicide Basta at field concentrations. By applying an antisense strategy towards caffeoyl CoA 3 - O-methyl-transferase (CCoAOMT), an enzyme induced during lignification, 11 of the selected transgenic alfalfa plants showed Klason lignin reduced up to 20%. This correlated with better digestibility, confirmed by "wet" analyses and NIRS. All transformants exhibited normal phenotype and fertility. The group has started an experimental program to investigate the possibility for production of a human lactoferin protein in alfalfa.

Laboratoire des Interactions Plantes-Microorganismes (LIPM) CNRS-INRA, Toulouse, France

T. Huguet* and his colleagues are developing a genetic approach for gene discovery based on the natural variations of *M. truncatula*. A number of molecular and genetic tools have been developed: microsatellite markers, recombinant inbred lines and genetic maps. Polymorphisms for traits of agronomic interest (nitrogen fixation, stem architecture, disease resistance, abiotic stress tolerance) have been identified in *M. truncatula*.

Pascal Gamas, Andreas Niebel, Laurence Godiard and colleagues study the genetic program activated in *Medicago truncatula* during symbiotic interactions with *Sinorhizobium meliloti*. Their studies are based upon a series of standard and subtractive cDNA libraries, and gene expression analyses on macro- / micro-arrays. Various candidate genes of interest which have been identified this way (particularly regulatory genes) are being characterized by reverse genetics approaches.

J. Dénarié, C. Gough, C. Rosenberg, F. Debelle and colleagues are involved in the study of the *M. truncatula* genome: they contribute to the construction of the physical map, to the anchoring of the physical map on the genetic map and to the sequencing of chromosome 5. They are making a genetic and molecular dissection in *M. truncatula* of symbiotic rhizobial and mycorrhizal infection and Nod factor signalling. Positional cloning of genes controlling symbiotic infection (nodule formation and mycorrhizal colonization) is in progress.

T. Timmers and his group are focusing on the cellular changes taking place during the interaction of *Medicago* with symbiotic partners, i.e. rhizobia and mycorrhizal fungi. Recent emphasis has been on the dynamics of the plant cytoskeleton using several fusions between cytoskeletal proteins and GFP, together with live cell imaging.

David Barker is investigating the *S. meliloti/M. truncatula* association through analysis of host gene expression in response to Nod factor signaling, and identification of regulatory elements in plant promoters specifically responsible for both Nod factor induction and expression during root hair infection. A study of arbuscular mycorrhizal/*M. truncatula* signaling during early stages of the interaction is making use of cell-specific ENOD-GUS reporter genes and fluorescent markers to identify intracellular rearrangements and modifications of the plant cell cytoskeleton prior to and during hyphal penetration. Both *Agrobacterium tumefaciens* and *A. rhizogenes* are being used to optimize transformation of *M. truncatula*. Construction of a large-scale transposon-tagged insertion library for *M. truncatula* is in progress.

Julie Cullimore, Jean-Jacques Bono and colleagues are studying receptors involved in Nod factor perception.

Laboratoire de Biotechnologie et d'Amélioration des Plantes (BAP), Ecole Nationale Supérieure d'Agronomie de Toulouse (ENSAT), Toulouse, France

M. Petitprez and his colleagues have started to study the induction of plant defense reactions in *M. truncatula* during plant-microbe interactions. A combination of transcriptomic, genetic and cellular approaches will be used to help to reveal if the basal level of plant defense genes are systemically or locally up-regulated or down-regulated during nodulation and if there is an effect on pathogenic or symbiotic (*Rhizobium*, mycorrhizae) interactions.

INRA, Unité de Génétique et d'Amélioration des Plantes Fourragères, Lusignan, France

Bernadette Julier* and Christian Huyghe* are carrying out several projects with alfalfa and *Medicago truncatula*. A genetic map of tetraploid alfalfa is being made, based on a F1 population produced by crossing two heretozygous plants selected from a Provence-type and a Flemish-type variety. AFLP markers and SSR markers, mainly derived from EST database of *M. truncatula* were used. The resulting map is considered to be saturated at about 92%. Based upon SSR markers, the co-linearity between tetraploid alfalfa and *M. truncatula* appears very good. In alfalfa QTLs for stem elongation rate and for *M. truncatula* QTLs for flowering date, plant structure, and stem elongation rate were mapped. Markers in candidate genes for aerial morphogenesis are being developed and mapped. SSR markers that were mapped in tetraploid alfalfa were used to assess the genetic variability within and among varieties, with one marker per chromosome. This survey showed that 1) each variety was at a panmictic equilibrium, 2) the allelic variability was very broad within every variety and was fairly similar among variety, 3) there was no narrowing of the variability as the breeding program progressed and 4) it was possible to differentiate most of the varieties with SSR markers.

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Daniele Rosellini*, Stefano Capomaccio, and Fabio Veronesi are continuing a collaboration with Wayne Parrott, University of Georgia (UGA), and Joe Bouton*, Noble Foundation, to develop acid soil-tolerant alfalfa. Some T_0 plants expressing bacterial citrate synthase had significant tolerance, and progeny are being tested in the greenhouse. Crosses with malate dehydrogenase-expressing plants have been made at UGA to stack the two transgenes. A cDNA-AFLP technique is being used to isolate genes involved in megasporogenesis. About 100 differentially expressed ESTs and 5 full-length cDNA have been identified that may be involved in the sterility trait. Attempts to transform chloroplasts have been unsuccessful. Research is planned to test co-transformation and non-antibiotic selective substances to avoid antibiotic resistance genes in engineered alfalfa.

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